

Hemoglobin Adducts of *N*-Substituted Aryl Compounds in Exposure Control and Risk Assessment

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Arylamines, nitroarenes, and azo dyes yield a common type of metabolite, the nitrosoarene, which produces a hydrolyzable adduct with protein and is closely related to the critical, ultimate toxic and genotoxic metabolite. The target dose as measured by hemoglobin adducts in erythrocytes reflects not only the actual uptake from the environment but also an individual's capacity for metabolic activation and is therefore an improved dosimeter for human exposure. The usefulness of hemoglobin adducts in molecular epidemiology is now widely recognized. With regard to risk assessment, many questions need to be answered. The described experiments in rats address some of these questions. The relationship between binding to hemoglobin in erythrocytes and to proteins in plasma has been found to vary considerably for a number of diamines. The fraction of hydrolyzable adducts out of the total protein adducts formed also varies in both compartments. This indicates that the kind of circulating metabolites and their availability in different compartments is compound specific. This has to do with the complex pattern of competing metabolic pathways, and the role of *N*-acetylation and deacetylation is emphasized. An example of nonlinear dose dependence adds to the complexity. Analysis of hemoglobin adducts reveals interesting insights into prevailing pathways, which not only apply to the chemical, but may also be useful to assess an individual's metabolic properties. In addition, it is demonstrated that the greater part of erythrocytes and benzidine-hemoglobin adducts are eliminated randomly in rats, i.e., following first-order kinetics.

Binding of *N*-Substituted Arenes to Blood Proteins

Since our first study on binding of arylamines to blood proteins (1) and the proposal that hydrolyzable sulfinic acid amides are formed by the reaction of nitroso derivatives with the sulfhydryl group of cysteine (2), we have investigated quite a number of chemicals in the rat (3-7). In most cases unlabeled compounds were used and hemoglobin (Hb) binding was of primary interest, so only the hydrolyzable fraction of hemoglobin adducts was reported. Occasionally, radiolabeled chemicals were available and enabled us to ask some additional questions that are relevant to assess the potential of using blood protein binding data for risk assessment. One aspect usually concerns the relationship between the protein target dose in erythrocytes and the tissue dose in liver and extrahepatic tissues. A clue to this complex problem may be obtained by comparing hemoglobin binding with binding to plasma proteins. Additional information may be obtained from the comparison of the hydrolyzable fractions of the adducts in both compartments. Some data are compiled in Table 1.

The data in Table 1 are arranged in the order of the hemoglobin binding index (HBI), which corrects for the different doses used and is defined as mmole/mole Hb/dose (mmole/kg). A plasma protein binding index (PPBI) is used accordingly. As demonstrated previously (4,8), the fraction of the administered dose that binds to hemoglobin varies over a wide range. With the exception of 3,3'-dichlorobenzidine, most of the hemoglobin binding is attributable to the hydrolyzable sulfinic acid amide adduct (70-90%), which indicates that reactive metabolites other than the nitrosoarenes are not available in the erythrocytes to a great extent. Binding to plasma proteins is highly variable, i.e., specific binding may be in the same range as to hemoglobin, or significantly lower or higher than that (ratio Hb:PP). Moreover, nonhydrolyzable adducts of unknown origin dominate.

Some points are notable. Nitrosobenzene is the exclusive adduct-forming metabolite of nitrobenzene in erythrocytes but seems to be also readily available in plasma (hydrolyzable fraction 98 and 59%, respectively). Protein-binding metabolites other than nitrosobenzene are available in plasma with acetanilide, possibly derived from *p*-hydroxy-acetanilid (hydrolyzable fraction 16% as compared to 59% with nitrobenzene). 4-Chloroaniline binds extremely well to hemoglobin. The nitroso derivative seems to be formed predominantly within the erythrocyte, and not much of it is available in plasma (ratio Hb:PP = 29). The differences between benzidine and 3,3'-dichlorobenzidine are also noteworthy. In both cases metabo-

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Table 1. Total binding of *N*-substituted arenes to blood proteins 24 hr after oral administration of the labeled compounds and the hydrolyzable fraction.

Compound administered	Dose, μmole/kg	Binding to				Ratio binding Hb:PP
		Hemoglobin		Plasma proteins		
		HBI	Hydrolyzable %	PPBI	Hydrolyzable %	
4-Chloroaniline	14	162	93	5.5	24	29.3
<i>trans</i> -4-Acetylamino stilbene	29	157	73	70	5	2.2
Nitrobenzene	200	73	95	9.3	58	7.6
Benzidine	1.1	56	84	111	ND	0.5
Acetanilid	150	12	88	4.8	16	2.5
3,3'-Dichlorobenzidine	50	6	32	36	26	0.2

Abbreviations: HBI, hemoglobin binding index; PPBI, plasma protein binding index; Hb, hemoglobin; PP, plasma protein; ND, not determined.

lites bind more extensively to plasma proteins than to hemoglobin (ratio Hb:PP 0.5 and 0.2, respectively). With 3,3'-dichlorobenzidine, a nitroso derivative contributes significantly to binding in plasma.

It may be inappropriate to try to correlate such findings with carcinogenicity at the present time for two reasons: first, a direct comparison of the carcinogenic potency in Wistar rats is not available; second, the data demonstrate that formation, distribution and, as a consequence, the target dose of reactive metabolites do not seem to be predictable from measuring a particular adduct in erythrocytes in case of *N*-substituted arenes. It remains to be further elucidated whether better conclusions can be drawn about the target dose in extrahepatic tissues when both protein binding in plasma and in erythrocytes are considered.

Elimination of Hemoglobin Adducts

To correlate protein binding data with genotoxic target doses, it is also necessary to consider the stability of adducts and their elimination kinetics. There is some confusion in the literature because the term "elimination half-life" is not consistent with a definite life span of erythrocytes. If erythrocytes are eliminated according to old age, their elimination rate together with that of Hb adducts should follow zero-order kinetics. However, exponential elimination of adducts has been described and consequently associated with chemical instability of the adducts (9).

We previously described an exponential elimination of Hb-benzidine adducts in rats with a half-life ($t_{1/2}$) of 11.5 days (10) and studied this problem recently in more detail (11). For this purpose, erythrocytes were labeled by injecting [^3H]diisopropyl-fluorophosphate (DFP) into 18 female Wistar rats. The animals were divided into two groups after 3 days; one received [^{14}C]-benzidine by gavage (2.5 μmole/kg), the other one served as a solvent control. Radioactivity of washed erythrocytes was determined five times up to 28 days after administration. From the semilogarithmic plot, an exponential decrease was delineated with $t_{1/2} = 14.4$ and 14.7 days, respectively, for the DFP label and $t_{1/2} = 14.4$ days for the benzidine label.

The experiment was repeated with 52 animals (2 for each time point) over 44 days. Although a linear regression could have been calculated with a satisfying correlation coefficient, this did not seem to be acceptable because a trend toward a decreasing slope for benzidine binding from early over middle to late time points was evident in the linear plot. In the semilogarithmic plot, on the other hand, it turned out that the slope became steeper for the period from 27 to 44 days. If the relationship recommended by the International Committee for Standardization in Haematology (12)

$$N(t) = N_0 \frac{e^{(-kt)} - e^{(-kT)}}{(1 - e^{(-kT)})}$$

(k = fraction of erythrocytes eliminated per day independent of age; T = life span of erythrocytes; N_0 = number of erythrocytes at time 0).

was used to fit the data, the values for k and T could be calculated. According to this equation, the upper limit of the life span of erythrocytes in these rats is 65 days, but in addition 3% per day of the erythrocytes are eliminated independent of age. This implies that two processes take place independently and that the elimination rate is satisfactorily described by an exponential function ($t_{1/2}$) up to 40 days. Also, in the second experiment described above, the benzidine label ($t_{1/2} = 16.5$ days) and the DFP label ($t_{1/2} = 18$ days) of washed erythrocytes were eliminated with the same rate.

The elimination rate of erythrocytes in rats has been measured by many authors and with different methods. Labeling with ^{59}Fe , ^{51}Cr , ^{32}P [DFP], and [^{14}C]glycine was used. Average life spans from 40 to 100 days were reported. In some more recent publications (13–16), elimination was measured up to 20 days, semilogarithmic plots were used, and first-order kinetics were assumed. Our values are in good agreement with those reported: 18.7 (15), 14.6 (16), and 13.5 days (17).

In summary, in contrast to mouse and man, random destruction of erythrocytes is faster in the rat, and the elimination can best be described initially by an exponential function. The results demonstrate that benzidine treatment does not alter the elimination rate of erythrocytes. In addition, a significant difference between the elimination rates of erythrocytes and the Hb adducts could not be demonstrated. The benzidine adduct is therefore considered stable *in vivo*.

Hemoglobin Binding of Diaminoarenes

Hemoglobin adduct levels vary widely between individuals. This may be partly due to differences in metabolic activation. *N*-Acetylation may play a particular role because notable differences occur between rapid and slow acetylators (18). Such a role became particularly apparent when we studied Hb binding of bifunctional *N*-substituted arenes in rats. Hydrolysis of the sulfinic acid amide adduct yields products in which the nonbinding nitrogen may either be acetylated or nonacetylated. Surprising differences were observed in this relationship (Table 2).

Benzidine is predominately activated by *N*-oxidation of the monoacetylated diamine. This is indicated by the ratio for the cleavage products diamine:monoacetyldiamine of 1:10. With

Table 2. Hemoglobin binding of bifunctional arylamines 24 hr after oral administration to female Wistar rats and the cleavage products obtained after hydrolysis of the adducts.

Compound administered	Dose, mmole/kg	HBI	Cleavage products		Ratio
			Diamine	Monoacetyldiamine	
Benzidine	0.5	21.3	2.4	18.9	0.1
3,3'-Dichlorobenzidine	0.5	3.5	2.0	1.5	1.3
3,3'-Dimethylbenzidine	0.5	2.9	1.9	1.0	1.9
3,3'-Dimethoxybenzidine	0.5	2.7	2.6	0.1	26.0
4,4'-Ethylenedianiline	0.5	18.1	14.7	3.4	4.3
4,4'-Oxydianiline	0.5	16.8	10.4	6.2	1.7
4,4'-Methylene-bis-2-chloroaniline	0.5	5.1	5.1	ND	—
4,4'-Methylenedianiline	0.5	3.7	3.1	0.6	5.2
	0.07	4.2	2.5	1.7	1.5
2,4-Diaminotoluene	0.25	ND			
	10 × 0.1 (19 days)	ND			
2,6-Diaminotoluene	0.25	0.2	ND	0.2	—
	10 × 0.1 (19 days)	0.4	ND	0.4	—

ND, not detected.

3,3'-dimethoxybenzidine, total binding is lower but this ratio is quite different (26:1). With 4,4'-methylene-bis-2-chloroaniline (MOCA), the acetylated diamine could not be detected at all. The other bridged derivatives (Table 2) form predominantly non-acetylated adducts. In contrast, the two isomeric diaminotoluenes seem to be activated only in the acetylated form.

Implications for risk assessment can be seen with benzidine. Benzidine is not a substrate for cytochrome P-450 or flavin monooxygenase (19). However, it can be oxidized by peroxidases to benzidine diimine, which is able to react with DNA (20). As a monoacetyl derivative, on the other hand, benzidine is a substrate for cytochrome P-450, and a single *N*-acetylation has been considered essential for the critical activating pathway (21). Benzidine and the three 3,3'-disubstituted congeners (Table 2) are classified as carcinogenic (22), but a direct comparison of the carcinogenic potency is not available. Mutagenicity has been compared in *Salmonella typhimurium*. In both strains, TA98 and TA100, mutagenicity decreased in the order 3,3'-dichlorobenzidine > 3,3'-dimethoxybenzidine > benzidine (23). The HBI would suggest quite another sequence of potency, with benzidine yielding the highest genotoxic target dose. Considering the differences in metabolic activation in the bacterial system even in the presence of liver enzymes and the *in vivo* situation, it seems plausible that the HBI reflects the presence of genotoxic metabolites *in vivo* better than the mutagenicity data.

Another aspect that may have to do with *N*-acetylation equilibria is the dose dependence of metabolic activation. We have studied the dose dependence of Hb binding of 3,3'-dichlorobenzidine, which is released from many industrial azo dyes after azo reduction, and observed significant deviations from a dose-proportionate increase (Fig. 1). The HBI for the hydrolyzable fraction increases from 0.5 at low doses to 2.7 at high doses in this study. This is almost completely due to an over-proportionate increase of the diamine within a narrow dose range around 0.1 mmole/kg. The ratio diamine:monoacetyldiamine increases clearly from 3 to 10 (Zwirner-Baier and Neumann, unpublished data).

Included in Figure 1 are data obtained with 4,4'-methylenedianiline (MDA) published by Bailey et al. (24). In contrast to our results for MDA (Table 2), these authors find more of the monoacetylated cleavage product than of the diamine, but they also observed a relative increase of the diamine fraction with

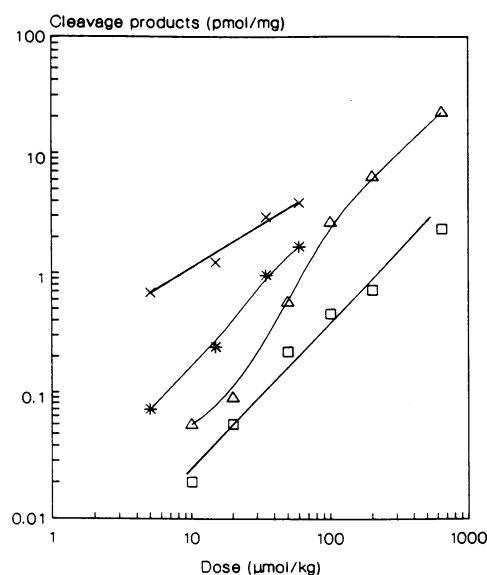


FIGURE 1. Dose dependence of hemoglobin binding. 3,3'-Dichlorobenzidine (DCB) was orally administered to female Wistar rats. Hemoglobin was isolated 24 hr later, and the two cleavage products obtained by hydrolysis were determined by HPLC with electrochemical detection. (Δ) DCB; (\square) monoacetyl-DCB. The data for ($\ast-\ast$) 4,4'-methylenedianiline (MDA) and ($\times-\times$) monoacetyl-MDA were taken from Bailey et al. (22).

dose (ratio diamine:monoacetyldiamine from 0.1 to 0.4). These data cannot readily be interpreted. Saturation of the *N*-acetylation capacity, however, does not seem to be the explanation.

Hemoglobin Adducts As Indicators of Metabolic Activation *in Vivo*

Dinitrotoluenes are of great practical importance as starting material for the production of diaminotoluenes, which are used in great quantities as intermediates in the synthesis of toluene diisocyanates, components in polyurethane production. In addition, both dinitrotoluenes and diaminotoluenes are found in the environment as waste of the former production of explosives. The

Table 3. Metabolic pathways and bioavailability of reactive metabolites as indicated by the formation of hemoglobin adducts.

Compound administered	Carcinogenic	Cleavage products indicating		
		N-Oxidation only	NO ₂ -Reduction	Acetylation
2,4-Diaminotoluene	+	ND		ND
2,4-Dinitrotoluene	+		4-A-2-NT (0.7) ^a	
2-Amino-4-nitro-toluene	+		2,4-DAT (0.9)	2-AA-4-AT (0.1)
2-Nitro-4-amino-toluene	?	2-N-4-AT (0.1)		
2,6-Diaminotoluene	—			2-AA-6-AT (0.2)
2,6-Dinitrotoluene	++		2-A-6-NT (1.0)	
			2,6-DAT (0.2)	
2-Amino-6-nitrotoluene	?	2-A-6-NT (0.4)	2,6-DAT (0.2)	2-AA-6-AT (0.1)

Abbreviations: A, amino; AA, acetyl amino; DA, diamino; N, nitro; T, toluene; ND, not detected.

^aHemoglobin binding index in parentheses.

2,4- and 2,6-isomers are most prevalent. Methods for bio-monitoring are desirable.

Practically all amino and nitro derivatives of toluene are mutagenic in *in vitro* tests with some differences in potency (25–27). The tests on carcinogenic potential, on the other hand, reveal striking differences. 2,6-Dinitrotoluene (2,6-DNT) is a strong carcinogen (28), whereas 2,6-diaminotoluene (2,6-DAT) is negative (29). 2,4-Dinitrotoluene (2,4-DNT) is significantly less carcinogenic than the 2,6-isomer, and it has even been suspected that impurities with the latter may explain the effect. In contrast, 2,4-diaminotoluene (2,4-DAT) is carcinogenic (30). These findings lead to the conclusion that expression of the genotoxic potential of these chemicals is markedly influenced by pharmacokinetic parameters in the complex *in vivo* situation.

In addition to the question of whether Hb binding can be used for exposure control, it was of interest to see whether the cleavage products from Hb adducts tell something about preferential metabolic pathways. Only parts of the metabolism of these compounds are known. It is assumed that a *N*-hydroxy-amino function has to be formed metabolically for genotoxicity. Thus, an amino group has to be *N*-oxidized or a nitro group has to be reduced to generate a proximate mutagen. Acetylation of an amino group can be another determinant. Because the hydrolyzable adduct of Hb is formed by the reaction of a nitroso group with a sulfhydryl group and releases an amino group, every cleavage product contains at least one amino function that indicates which nitrogen was involved in metabolic activation and adduct formation.

The results that were obtained by hydrolyzing Hb adducts 24 hr after feeding the various chemicals to female Wistar rats are summarized in Table 3. Hydrolyzable Hb adducts were found with all chemicals tested, except with 2,4-DAT. The HBI values are comparatively low, so the analysis of human blood samples will require extremely sensitive methodology. In addition, the situation is complicated by the complex pattern of cleavage products of these bifunctional substances. A simple correlation between carcinogenicity and Hb binding could not be detected in this study. This may be partly due to the fact that another possibly relevant metabolic pathway is not controlled with this method: the oxidation of the methyl group. 2,4-Dinitrobenzylalcohol-glucuronide and 2,4-dinitrobenzoic acid are major metabolites of 2,4-DNT in rat urine (31). 4-Acetylamino-2-aminobenzoic acid was described as a metabolite of 2,4-DAT (32). Such a C-oxidation product, however, was not observed with 2,6-DAT (33). Analysis of urinary metabolites did not indicate the formation of *N*-oxidation products in both studies with diamino-

toluenes. Because 2-acetylamino-6-aminotoluene has been identified after hydrolysis of Hb from 2,6-DAT-treated animals, it is now evident that *N*-oxidation occurs *in vivo* (Table 3). With a limited amount of ¹⁴C-labeled 2,4-DAT and 2,6-DAT (kindly provided by M. Cunningham), we were able to measure total binding to Hb and plasma proteins as well as total tissue concentration in lung, liver, kidney, spleen, and adrenals. With 2,6-DAT, all values were on average one-third of those obtained with 2,4-DAT, i.e., tissue doses are lower with the non-carcinogenic 2,6-DAT (Zwirner-Baier and Neumann, unpublished data). It is interesting to note, however, that reactive metabolites bind to blood proteins with both chemicals, but with 2,4-DAT only nonhydrolyzable Hb adducts are formed, and with 2,6-DAT only a small fraction of the bound material is hydrolyzable. The origin of nonhydrolyzable adducts remains to be studied. Phenolic metabolites are possible precursors in addition to *N*-oxidation products and benzylic alcohol or benzaldehyde derivatives. One such metabolite, 5-hydroxy-2-acetylamino-6-aminotoluene, is mutagenic in *S. typhimurium* TA 98 in the presence of S9-mix (33).

Nevertheless, some interesting conclusions can be drawn about prevalent metabolic pathways. With 2,6-DAT, acetylation of one amino group is a prerequisite for *N*-oxidation of the other. An *N*-oxidation product of 2,4-DAT could not be demonstrated. In 2,4-DNT only the nitro group in the *para* position is reduced, although a nitro group *ortho* to the methyl group is accessible to reduction. With 2,6-DNT both nitro groups are reduced. In 2-amino-4-nitrotoluene, which is also classified as carcinogenic (22), the *para* nitro group is more readily reduced than the *ortho* amino group oxidized. In 2-nitro-4-aminotoluene it is also the *para* substituent that is preferentially metabolized. In the *ortho*-disubstituted 2-amino-6-nitrotoluene both functions are metabolized. In other words, an amino function in *ortho* position to the methyl group can in principle be oxidized as well as acetylated *in vivo*. A nitro group can be reduced in this position. Both functional groups are preferentially metabolized in the *para* position, regardless of the second substituent in the *ortho* position. The results may also indicate that the nitro groups are not necessarily reduced to the amines before macromolecular binding.

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